Amendments to the specification

Please replace the description of Fig. 7 starting on page 26 with the following amended description:

~~Fig. 7:~~ Figs. 7a-7b: Time course of phosphorylation of bacterially expressed human isoform htau23 with the brain kinase activity and corresponding autoradiogram.

~~(a)~~ (7a) SDS-PAGE of htau23 after incubation with the kinase between 0 and 24 hours, as indicated. The unphosphorylated protein is a single band of M_{ro}=48 kD (lane 1). Lanes 3-14 show that phosphorylation leads to a progressive shift to higher M_r with well defined intermediate stages. The even lanes (numbered 4, 6, etc. below Fig. 1b) are observed in the presence of 10 µM okadaic acid (OA) (labeled "+" below Fig. ~~1a~~ 7a). the odd lanes (3,5, etc. labeled "-") are without okadaic acid. The first stage takes about 2 hours (shift to a new M_{r1}=52 kD), the third is finished around time 24 hours (M_{r3}=56 kD); no further shift is observed during the subsequent 24 hours. Lane 2 shows a mutant that is not of significance in this context.

~~(b)~~ (7b) Autoradiogram of ~~(a)~~ (7a). The quantitation of the phosphate incorporated (mol P_i/mol protein) in this experiment was as follows (-OA/+OA): 30 min (0.5/1.0), 60 (0.7/1.4), 120 min (1.0/2.0), 10 hours (2.0/3.0), 24 hours (3.2/4.0).

Please replace the description of Fig. 8 starting on page 27 with the following amended description:

~~Fig. 8:~~ Figs.8a-8b ~~(a)~~ (8a) SDS gel showing the time course of phosphorylation of htau23 similar to that of Fig. 1a, but with 10 μM okadaic acid throughout;

~~(b)~~ (8b) immunoblot of ~~(a)~~ (8a) with the monoclonal antibody SMI34. The antibody recognizes the protein only in the second and third stage of phosphorylation, but not in the first.

Please replace the description of Fig. 9 starting on page 27 with the following amended description:

~~Fig. 9:~~ Figs. 9a-9c: Binding of tau isoforms to microtubules before and after phosphorylation.

~~(a)~~ (9a) SDS gel of a binding experiment, illustrated for the case of the tau isoform htau40 (whose band is clearly separated from that of tubulin (T) so that both components can be shown simultaneously, without having to remove tubulin by a boiling step). The top line indicates pellets (P) or supernatants (S), with or without phosphorylation for 24 hours (+ or $-P_i$). Lanes 1-4, 20 µM tau protein (total concentration), phosphorylated (lanes 1, 2) or not (lanes 3, 4). The comparison of lanes 1 and 2 shows that most of the phosphorylated protein is free (S), while only a small fraction is bound to the microtubules (P). Lanes 3 and 4 show that in the unphosphorylated state about half of the protein is bound, the other half free (note also that the phosphorylated protein bands, lanes 1, 2, are higher in the gel than the unphosphorylated ones, lanes 3, 4,

similar to Fig. 1). Lanes 5-8, similar experiment with 15 μM htau40. Lanes 9, 10 show the case of 10 μM phosphorylated protein. Lanes 11-15 are for density calibration with known amounts of htau40 (15, 10, 7.5, 5, and 2.5 μM, resp.).

~~(b)~~ (9b) Binding curves of htau23 and ~~(c)~~ (9c) htau34 to microtubules before (circles) and after 24 hour phosphorylation (triangles); these curves were derived from SDS gels similar to that of Fig. 3a. Polymerized tubulin is 30 μM. Fitted dissociation constants K_d and stoichiometries are as indicated. In each case the most dramatic effect is on the number of binding sites which decrease about three-fold upon phosphorylation, from around 0.5 (i.e. one tau for every two tubulin dimmers) down to about 0.16 (one tau for six tubulin dimmers). Note that the binding of unphosphorylated 4-repeat isoforms (such as htau34) is particularly tight (K_d round 1-2 μM).

Please replace the description of Fig. 12 starting on page 29 with the following amended description:

~~Fig. 12:~~ Figs. 12a-12d: SDS-PAGE and immunoblots of tau protein from Alzheimer and normal human brain with antibodies SMI33, SMI31, SMI34.

~~(a)~~ (12a) Lane, 1, SDS-PAGE of tau protein from a normal human control brain showing 5-6 bands between M_r55 and 65 kD (somewhate lower than the PHF tau of lane 3). Lane 2, normal human tau after phosphorylation with kinase activity, resulting in an upward shift of all bands. Lanes 3, 4, immunoblot of PHF tau with antibody 5E2

which recognizes all tau isoforms independently of phosphorylation (Kosik et al., Neuron 1 (1988), 817-825). Lane 3, PHF tau as isolated from an Alzheimer brain; lane 4, after dephosphorylation with alkaline phosphatase. Note that the bands of the dephosphorylated protein are shifted down on the gel.

~~(b)~~ (12b) Immunoblot of ~~(a)~~ (12a) with SMI33. The antibody recognizes normal human tau (lane 1), and PHF tau after dephosphorylation (lane 4).

~~(c)~~ (12c) Immunoblot of ~~(a)~~ (12a) with SMI31. Note that the antibody recognizes normal human tau after phosphorylation, and PHF tau in its natural state of phosphorylation (lanes 2, 3).

~~(d)~~ (12d) Immunoblot of ~~(a)~~ (12a) with SMI34. This antibody recognizes normal human tau only after phosphorylation (lane 2), and PHF tau (lane 3).

Please replace the description of Fig. 13 starting on page 30 with the following amended description:

~~Fig. 13:~~ Figs. 13a-13h: Time course of phosphorylation of bacterially expressed human isoform htau23 (similar to previous figure) and immunoblots with antibodies SMI33, SMI31, SMI34, TAU1, and AT8.

~~(a)~~ (13a) SDS-PAGE, phosphorylation times 0-24 hours, showing the successive M_r SHIFTS.

~~(b-f)~~ (13b-13f) Immunoblots with SMI31, SMI33 and TAU1 recognize htau23 fully up to the end of

stage 1 (2 hours), but the epitope becomes blocked during the second stage. Antibodies SMI31, SMI34, and AT8 are complementary in that they recognize the protein only in the second and third stage of phosphorylation.

~~(g-h)~~ (13g-13h) Immunoblot of htau34 with SMI33 and SMI310 which recognize the protein from the stage 2 phosphorylation onwards, similar to SMI31.

Please replace the description of Fig. 14 starting on page 31 with the following amended description:

~~Fig. 14:~~ Figs. 14a-14d: SDS-Page of tau and several constructs, and immunoblots with the antibodies SMI33, SMI31, and SMI34.

~~(a)~~ (14a) SDS-PAGE. Lanes 1 and 2: Construct K10 before and after phosphorylation with the kinase for 24 hours. Lanes 3 and 4: Construct K17 before and after phosphorylation. Lanes 5 and 6: Construct K19 before and after phosphorylation. All constructs except K19 show a shift upon phosphorylation. With K10 one observes three shifted bands, with K17 there is only one shifted band.

~~(b)~~ (14b) Immunoblot of ~~(a)~~ (14a) with SMI33: The antibody recognized only K17 in the unphosphorylated form (lane 3), suggesting that the epitope lies before the repeats.

~~(c)~~ (14c) Immunoblot of ~~(a)~~ (14a) with SMI34. the antibody recognizes K10 and K17 in the

phosphorylated form (only top bands, lanes 2, 4). The antibody does not recognize K19 (the repeat region), but requires sequences on both the N-terminal and C-terminal side of the repeats. The epitope is therefore non-contiguous (conformation-dependent).

~~(d)~~ (14d) Immunoblot of ~~(a)~~ (14a) with SMI31. The antibody recognizes only the top band of the phosphorylated K10 (lane 2), suggesting that the epitope lies behind the repeat region.

Please replace the description of Fig. 16 starting on page 31 with the following amended description:

~~Fig. 16:~~ Figs. 16a-163 SDS gel of htau40 and the point mutants of Fig. 15, and immunoblots with antibodies SMI33, SMI31, and SMI34.

~~(a)~~ (16a) Lanes 1-8, SDS gel of htau40 and its mutants KAP235, KAP396, and KAP235/396 in the unphosphorylated and phosphorylated form (+).
In each case phosphorylation leads to an upward shift in the SDS gel.

~~(b)~~ (16b) Blot of ~~(a)~~ (16a) with SMI33. The antibody response is strongly reduced when S235 is mutated, both in the dephosphorylated and phosphorylated state (lanes 3+4, 7+8). This indicates that the (dephosphorylated) first KSP motif is part of the epitope of SMI33. When S396 is mutated to A the behavior is similar to the parent molecule, i.e. strong antibody response in the dephosphorylated state, no reaction in the

phosphorylated state, so that S396 does not contribute to the epitope of SMI33.

~~(c)~~ (16c) Blot of ~~(a)~~ (16a) with SMI31. The antibody recognizes htau40 and all mutants in the phosphorylated form (lanes 2, 4, 6, 8). This shows that phosphorylation of the two KSP motifs is not the main determinant of the epitope.

~~(d)~~ (16d) Blot of ~~(a)~~ (16a) with SMI34. The reaction is similar to SMI31 but more pronounced, again indicating that the two KSP motifs are not essential.

Please replace the description of Fig. 17 starting on page 32 with the following amended description:

~~Fig. 17:~~ Figs. 17a-17f: Deletion mutants of tau and their antibody response. ~~(a)~~ (17a) SDS gel of constructs containing only two repeats (K5-K7) or one repeat (K13-K15), before and after phosphorylation. ~~(b)~~ (17b) Immunoblot of ~~(a)~~ (17a) with SMI34. Note that the antibody recognizes all phosphorylated proteins (K7 only weakly). ~~(c)~~ (17c) Immunoblot of ~~(a)~~ (17a) with SMI31. Note that the antibody recognizes the phosphorylated two-repeat molecules (K5-K5), but not the one-repeat molecules (K13-K15). Lanes 7 and 8 show htau40 as a control. ~~(d)~~ (17d) SDS gel of constructs K2, K3M, and K4, before and after phosphorylation. ~~(e)~~ (17e) Blot of ~~(d)~~ (17d) with SMI34, recognizing only K4 phosphorylated. ~~(d)~~ (17d) Blot of (d) with SMI31, recognizing only K2 phosphorylated.

Please replace the description of Fig. 20 starting on page 33 with the following amended description:

~~Fig. 20:~~ Figs. 20a-20c: SDS PAGE (4-20%) and gel chromatography of tau constructs and cross-linked products. Gels a and c were run in reducing conditions (3mM DTT in sample buffer), gel b in non-reducing conditions (except lane 1 with 3 mM DTT in sample buffer).

~~(a)~~ (20a) Constructs T8R-1, Htau23 and K12. Molecular weight markers are given on the left.

~~(b)~~ (20b) Construct K12 and cross-linked products. Cross-linking occurs spontaneously in the absence of DTT; it can be prevented by DTT, or induced by addition of PDM or MBS. Aggregation products are labeled on the right (monomers, dimmers, trimers, tetramers etc.).

~~(c)~~ (20c) Silver stained SDS gel of a Superose 12 gel filtration run of K12 cross-linked by PDM. The dimmers (top band) elute before the monomers. Fractions 16 and 17 were used for electron microscopy.

~~(d)~~ (20d) Elution profile of Superose 12 gel filtration of construct K12 monomers and dimmers cross-linked with PDM. The elution positions of calibration proteins are plotted against their effective hydrated Stokes radii on a logarithmic scale (right axis).

~~(e)~~ (20e) CD spectrum of construct K12 (8 mg/ml in 40 mM HEPES pH 7.2, path length 0.01mm). There is significant α-helical or β-sheet structure.

Similar spectra are obtained with other constructs as well as with full length tau.

Please replace the description of Fig. 21 starting on page 34 with the following amended description:

~~Fig. 21:~~ Figs. 21a-21c: Synthetic paired helical filaments from construct K12.

~~(a)~~ (21a) A tangle of synthetic PHFs from K12 (crossover period of ≈ 70-75 nm indicated by arrowheads). The construct was expressed and purified by the methods described previously (Steiner et al.). It was dialysed against 0.5 M Tris-HCl, with pH values between 5.0 and 5.5. The solution was negatively stained with 2% uranyl acetate.

~~(b) and (c)~~ (21b-21c) Single fibers of synthetic paired helical filaments made from construct K12. Note the crossover repeats (arrowheads) and the rod-like particles of lengths around 100 nm (c, middle). Bar = 100nm.

Please replace the description of Fig. 22 starting on page 35 with the following amended description:

~~Fig. 22:~~ Figs. 22a-22c: Synthetic paired helical filaments from K12 dimers cross-linked with PDM and negatively stained with 1% phosphotungstic acid (micrographs provided by M. Kneil). Bar = 100nm.

Please replace the description of Fig. 23 starting on page 35 with the following amended description:

~~Fig. 23:~~ Figs. 23a-23b: Paired helical filaments from Alzheimer brain (micrographs provided by Dr. Lichtenberg-Kraag).

~~(a)~~ (23a) PHFs from neurofibrillary tangles, prepared after Wischik et al., stained with 1% phosphotungstic acid. This preparation contains homogeneous long filaments which still retain their pronase sensitive "fuzzy coat." The crossover repeat is 75-80 nm, the width varies between a minimum of about 10 nm and a maximum of 22 nm.

~~(b)~~ (23b) PHFs prepared after Greenberg & Davies. This preparation results in soluble filaments of shorter length than in ~~(a)~~ (23a) and is more heterogeneous. (1) is a paired helical filament with a 72 nm repeat and a width varying between 8 and 18 nm; (2) is a straight filament of 8 nm width; (3) is a twisted filament with a particularly wide diameter (up to 25 nm); (4) is a straight filament with a wide diameter (18 nm); (5) is a twisted rod-like particle about 80 nm long, equivalent to about one crossover period. In many cases the particles appear to have broken apart across the filament, e.g. the two rods labeled (4), the twisted filament of (3) and the short stub to the right of it, or the two straight rods above particle (3). Bar = 100 nm.

Please replace the description of Fig. 24 starting on page 36 with the following amended description:

~~Figs. 24:~~ Figs. 24a-24e: Electron micrographs of tau isoform htau23 and construct T8R-1 prepared by glycerol spraying and metal shadowing

~~(a)~~ (24a) monomers of htau 23,

~~(b)~~ (24b) dimmers of htau23,

~~(c)~~ (24c) monomers of T8R-1,

~~(d)~~ (24d) folded forms of T8R-1 (hair-pin folds showing intramolecular antiparallel association),

~~(e)~~ (24e) dimmers of T8R-1. For lengths see Table 1 and Fig. 7. Interpretative diagrams are shown on the right. Bar = 50 nm.

Please replace the description of Fig. 25 starting on page 36 with the following amended description:

~~Fig. 25:~~ Figs. 25a-25h: Length histograms of tau constructs and dimers.

Please replace the description of Fig. 26 starting on page 36 with the following amended description:

~~Fig. 26:~~ Figs. 26a-26h: Electron micrographs of constructs K11 and K12.

~~(a)~~ (26a) Monomers of K11,

~~(b)~~ (26b) dimmers of K11

~~(c)~~ (26c) Tetramers of K11 formed by longitudinal association of two dimmers.

~~(d)~~ (26d) Monomers of K12,

~~(e)~~ (26e) Dimers of K12,

~~(f)~~ (20f) tetramers of K12. Bar = 50 nm.

Please replace the description of Fig. 27 starting on page 36 with the following amended description:

~~Fig. 27:~~ Figs. 27a-27b: ~~(a)~~ (27a) K12 dimers cross-linked by PDM (i.e. Cys322 to Cys322); ~~(B)~~ (27b) K12 dimers cross-linked by MBS (i.e. Cys322 to nearby Lys). Bar = 50 nm.

Please replace the description of Fig. 28 starting on page 37 with the following amended description:

~~Fig. 28:~~ Figs. 28a-28d: Antibody labeling of htau23, K12 and cross-linked products thereof.

~~(a)~~ (28a) htau23 dimers with an antibody at one end (left) and with an antibody at each end (right) demonstrating that antiparallel dimerization of htau23;

~~(b)~~ (28b) K12 dimers with an antibody at one end (left), with antibodies at both ends (middle) and presumable tetramers with antibodies at the free ends (right indicating that this type of association blocks the epitpe;

~~(c)~~ (28c) K12 dimers cross-linked with PDM, with an antibody at one end (left), with antibodies at each end (middle) and a tetramer with antibodies at the free ends (right);

~~(a)~~ (28d) K12 dimers cross-linked by MBS with an antibody at one end (left), with antibodies at each end (middle) and a tetramer with antibodies at the free ends (right).
Bar = 50nm.

Please replace the description of Fig. 29 starting on page 37 with the following amended description:

~~Fig. 29:~~ Figs. 29a-29g: Time course of phosphorylation of htau40 by GSk3 and immune response. ~~(1)~~ (29a) SDS-PAGE of htau40 after incubation with the kinase between 0 and 20 hours at 37°C. The minor lower band in lane 1 is a fragment. Note the progressive shift to higher Mr values, similar to the effects of brain extract and MAP kinase. ~~(2)~~ (29b) Autoradiography. ~~(3)~~ (29c) Immunoblot with the antibody TAU1 whose reactivity is lost after ≈2 h (following the phosphorylation of S199 and S202) (29d). ~~(5)~~ (29e) Immunoblot with antibody SMI34 (conformation sensitive and against phosphorylated Ser). ~~(6)~~ (29f) Blot with SMI31 (epitope includes phosphorylated S396 and S404). ~~(7)~~ (29g) Blot with antibody SMI33 which reuires a dephosphorylated S235. There are some differences with respect to phosphorylation by MAP kinase or the brain extract. The SMI33 staining persists for a long period, suggesting that Ser235 is only slowly phosphorylated by GSK3. The staining of SMI31 appears very quickly, before that of AT8 or SMI34, showing that S396 and S404 are among the earliest targets of GSK3.

Please replace the description of Fig. 30 starting on page 38 with the following amended description:

~~Fig. 30:~~ Figs. 30a-30b: Mobility shift of htau23 versus mutant htau23/A404 upon phosphorylation with GSK3. ~~Top,~~ Fig. 30a, SDS gel, ~~bottom,~~ Fig. 30b, autoradiogram. Lanes 1-3, htau23 unphosphorylated and phosphorylated for 2 or 20 hours. Note the pronounced shift and the clear incorporation of phosphate. Lanes 4-6, mutant

Ser404-Ala, unphosphorylated and phosphorylated for 2 and 20 hours. The shift after 2 hours is much smaller and the degree of phosphorylation much lower. This shows that the first strong shift and phosphorylation is at Ser404, similar as with MAP kinase and the brain extract kinase activity.

Please replace the description of Fig. 32 starting on page 38 with the following amended description:

~~Fig. 32:~~ Figs. 32a-32d Copolymerization of MAP kinase and GSK3 with porcine brain microtubules. ~~(a)~~ (32a) SDS gel of microtubule purification stages. Ex = brain extract, supernatant after first cold spin. S = supernatant of first hot spin = tubulin and MAPs not assembled into microtubules after warming to 37°C; P = pellet of redissolved microtubules. The other lanes (S, P) show two further cycles of assembly and disassembly by temperature shifts (last pellet of microtubule protein was concentrated). ~~(b)~~ (32b) Blot with anti-MAP kinase, showing mainly the p42 isoform and some of the p44 isoform. ~~(c)~~ (32c) Blot with anti-GSK3β; note that this antibody shows some cross-reactivity with GSK3α. The blots show that both kinases and their isoforms co-purify with the cycles of microtubule assembly.

Please replace the description of Fig. 33 starting on page 39 with the following amended description:

~~Fig. 33:~~ Figs. 33a-33b: ~~(a)~~ (33a) Identification of GSK3α and β in normal and Alzheimer brain extracts. M = markers, lane 1, SDS gel of normal

brain extract, lane 2, immunoblot with anti-GSK3α; lane 3, immunoblot with anti-GSK3β (with some crossreactivity to α). Lanes 4 and 5, same blots with Alzheimer brain extracts.

Please replace the description of Fig. 35 starting on page 39 with the following amended description:

~~Fig. 35:~~ Figs. 35a-35b: ~~(a)~~ (35a) Diagram of htau23 and point nutants used in this invention. ~~(b)~~ (35b) Binding curves of htau23 and its point mutants to microtubules, unphosphorylated and phosphorylated with brain extract. The top and bottom curves show unphosphorylated and phosphorylated wild type htau23, the other curves are after phosphorylation. Mutants are (from top to bottom: Ser262-Ala, Ser235-Asp/Ser396-Asp, Ser404-Ala, Ser202-Ala. the mutation at Ser262 nearly eliminates the sensitivity of the tau-microtubule interaction to phosphorylation. These curves were derived from quantitating SDS gels by densitometry (see Example 6). Polymerized tubulin is 30 μM. The fitted stoichiometries n (= tau/tubulin dimmer) and binding constants K_d (μM) are:

htau23wt non-phos. (n=0, 49, K_d=2.5); A262 phos. (n=0.45, K_d=5.3); D235/D396 phos. (n=0.32, K_d=7.5); A404 phos (n=0.32, K_d=9.3); A202 phos. (n=0.31, K_d=9.4); htau23wt phos. (n=0.16, K_d=4.9).

Please replace the description of Fig. 37 starting on page 40 with the following amended description:

~~Fig. 37:~~ Figs. 37a-37b ~~(a)~~ (37a) Diagram of total mutant AP18. Al Ser-Pro Thr-Pro are replaced by Ala-Pro.

In addition, Ser262 and 356 are mutated into Ala. In the mutant AP17 Ser 262 and Ser356 remain unchanged. ~~(b)~~ (37b) Binding curves of htau 23 and the "total" mutants AP17 and AP18 to microtubules without or with phosphorylation by brain extract. Top, unphosphorylated htau23 (filled triangles); middle, phosphorylated AP18 (circles), the two bottom curves are phosphorylated AP17 (open squares) and htau23 (open triangles). The difference in behavior between AP17 and AP18 is due to the phosphorylation of Ser262 in AP17. Fitted stoichiometries and binding constants are: htau23wt non-phos. (n=0.49, K_d=2.5); AP18 phos (n=0.48, K_d=6.1); Ap17 phos (n=0.18, K_d=6.6); htau23wt phos. (n=0.16, Kd=4.9).

Please replace the description of Fig. 38 starting on page 41 with the following amended description:

~~Fig. 38:~~ Figs. 38a-38d: Preparation of the kinase from porcine brain by chromatographic steps. ~~(a)~~ (38a) Mono Q HR 10/10 FPLC. The phosphorylation of recombinant human tau 34 and construct AP17 is shown on the y-axis as moles P_i transferred per mole of tau. Fractions which decrease the binding of tau to MT elute around fraction 12, 20 and 30, the peaks around fractions 20 and 30 being the most effective. ~~(b)~~ (38b) Fractions 28-32 from Mono Q were gel filtrated on a Superdex G-75 HiLoad 16/60 column. The column was calibrated with standard proteins as shown by the filled symbols: Ribonuclease, 14 kDal; chymotrypsinogen A, 25 kDal; ovalbumin, 43 kDal; bovine serum albumin, 67 kDal. Molecular weight is indicate on

the right y-axis on a logarithmic scale. The phosphorylation of htau34 and construct K18 is shown on the left y-axis. The highest activity elutes at a Mr of approx. 35 kDal. ~~(c)~~ (38c) Fractions 17-23 from the gel filtration column were pooled and rechromatographed on a Mono Q HR 5/5 column. Fraction 10 was used for binding studies. ~~(d)~~ (38d) SDS-gel showing the main purification stages. M: Marker proteins; lane 1, whole brain extract lane 2, Mono Q HR 10/10 FPLC, fraction 30; lane 3, Superdex gel filtration, fraction 22; lanes 4-5, Mono Q HR 5/5 FPLC, fractions 10 and 9. Lane 5 shows the purified 35 kDal band and a trace at 41 kDal.

Please replace the description of Fig. 39 starting on page 42 with the following amended description:

~~Fig. 39:~~ Figs. 39a-39c: SDS gel and in-gel assay of kinase activity (for details see Example 11). ~~(a)~~ (39a) 7-15% silver stained SDS gel of fractions 9-11 (lanes 1-3) of second Mono Q run (see Fig. 38c). ~~(b)~~ (39b) Autoradiogram of an in-gel experiment, with tau construct K9 (= four repeats plus C-terminal tail of tau) in the gel and 5 µl each of fractions 9-11 (lanes 1-3). ~~(c)~~ (39c) Autoradiogram of control gel containing no tau protein and showing no autophosphorylation of the Mono Q fractions. Note that specific kinase activities are difficult to quantify from these gels since the renatured protein tends to diffuse out of the gels; this is especially true of the 37 kDal band.

Please replace the description of Fig. 40 starting on page 42 with the following amended description:

~~Fig. 40:~~ Figs. 40a-40b: Effect of phosphorylation of tau by 35 kDal kinase on gel shift and microtubule binding. ~~(a)~~ (40a) SDS gel of htau23 and constructs phosphorylated by several kinases. M, marker proteins. Lanes 1 and 2, htau23 without and with phosphorylation by 35 kDal kinase. Lanes 3 and 4, same experiment with point mutant htau23 (Ser409-Ala) (no shift); lanes 5 and 6, point mutant htau23 (Ser416-Ala) (only part of the protein phosphorylated, but otherwise same shift as in lane 2); lanes 7 and 8, point mutant htau23 (Ser404-Ala) (same shift as lanes 2 and 6). The mutants show that the 35 kDal kinase induces a shift by phosphorylating Ser409. Note that Ser404 is the target of MAP kinase, Ser416 of CaM kinase (Steiner et al., 1990, ibid.), and Ser409 and Ser416 of PKA each of which induces a shift. Lanes 9-11 show a comparison of the shifs induced in htau23 by the diffeent kinases (CaM kinase, PKA and MAP kinase). The shifts induced by PKA (lane 10) is the same as that of the 35 kDal kinase, and that MAP kinase produces by far the largest shift, typical of the Alzheimer-like state of tau. The bars on the right indicate the shift level; from bottom to top, unphosphorylated htau23 (control), CaM kinase shift level, PKA shift level, MAP kinase shift level. All shift sites are near the C-terminus. ~~(b)~~ (40b) Binding curves of htau23 and the mutants Ser262-Ala to microtubules without or with phosphorylation by the 35 kDal kinase (Mono Q fraction 10, 20 hours). Top, unphosphorylated htau23 (open circles, n=0.49,

K_d=2 5 μM); middle, phosphorylated mutant (squares, n=0.44, K_d=11.6 μM); bottom, phosphorylated htau23 (filled circles, n=0.21, K_d=8.8 μM). In the absence of Ser262 the reduction in stoichiometry is 0.05; with phosphorylated Ser262 it is 0.28.

Please replace the description of Fig. 42 starting on page 43 with the following amended description:

~~Fig. 42:~~ Figs. 42a-42d ~~1.~~ Dephosphorylation ("dephos.") of p32-marked htau40 ("ht40^{32}p") with different PPases. Autodigraphs of 7-15% SDS gradient gels.

~~Fig. 1:~~ Autoradiographs of 7-15% SDS gradient gels.

~~A.~~ 42a. Dephos. with PP2a H-isoform (10μg/ml)

Lane 1: ht40P before dephos.

Lane 2: 10 min dephos.

Lane 3: 30 min. dephos.

Lane 4: 12 min dephos.

~~B.~~ 42b. Dephos. with PP2a M-isoform (10μg/ml),

Lanes 1-4: see ~~A.~~ 42a.

~~C.~~ 42c. Dephos. with PP2a L-isoform (10μg/ml),

Lanes 1-4: see ~~A.~~ 42a.

~~D.~~ 42d. Dephos. with catalytic subunit of PP1 (500 U/ml),

Lanes 1-4: see ~~A.~~ 42a.

Please replace the description of Fig. 43 starting on page 44 with the following amended description:

~~Fig. 43:~~ Figs. 43a-43e ~~2.~~ Dephosphorylation with PP2a-H: disappearing of phosphorylation dependent antibody epitopes

~~A.~~ 43a. SDS-PAGE (7-15%).

Lane 1:ht40P before dephos.

Lane 2: 10 min dephos.

Lane 3: 30 min dephos.

Lane 4: 120 min dephos.

Lane 5: 5h dephos.

Lane 6: 16h dephos.

~~B.~~ 43b. Autoradiographs

~~C.~~ 43c. Immunoblot AT-8

~~D.~~ 43d. Immunoblot Tau-1A

~~E.~~ 43e. Immunoblot SMI-33

Please replace the description of Fig. 44 starting on page 44 with the following amended description:

~~Fig. 44:~~ Figs. 44a-44b: Kinetics of dephos. with PP2a-H

~~a.~~ 44a. time course of dephos. of ht40P with different concentrations of PP2a

~~b.~~ 44b. variation in the ht40P-concentration: Michaelis-Menten-Diagramm.

Please replace the description of Fig. 45 starting on page 44 with the following amended description:

~~Fig. 45:~~ Figs. 45a-45c: Preparation of the 70 kDal kinase which phosphorylates the two IGS motifs and the two CGS motifs of tau protein (Serines 262, 293, 324, 409). The kinase strongly reduces the affinity of tau for microtubules.

~~(a)~~ (45a) Chromatography on S-Sepharose. Kinase activity elutes at 250 mM NaCl.

~~(b)~~ (45b) Chromatography on heparin agarose. Kinase activity elutes at 250 mM NaCl.

~~(c)~~ (45c) Gel filtration on Superdex G-75. Kinase activity elutes at 70 kDal.

Please replace the description of Fig. 46 starting on page 45 with the following amended description:

~~Fig. 46:~~ Figs. 46a-46d: Time course of phosphorylation of htau40 with cdk2/cyclin A. Lanes 1-9 correspond to time points 0, 10, 30, 90 min, 3, 6, 10, 24 hours, and 0 min (the 0 min lanes are the control).

~~(a)~~ (46a) SDS polyacrylamide gel electrophoresis, showing the shift of the protein upon phosphorylation.

~~(b)~~ (46b) Autoradiogram showing increasing incorporation of phosphate.

~~(c)~~ (46c) Immunoblot with TAU-1 antibody which recognizes only unphosphorylated Ser 199 and Ser 202.

~~(d)~~ (46d) Immunoblot with AT-8 antibody which recognizes these two serines in a phosphorylated state, as well as Alzheimer tau.

Please replace TABLE 1 on page 87 with the following TABLE 1 containing appropriate font size:

TABLE 1: Interactions of tau constructs with antibodies in the phosphorylated or unphospborylated state (+ or -). The staining on immunoblots ranges from very weak (x), to very strong, xxx.

Construct	phosph. +/-	SM133	SM131	SM134
htau40	-	xxx		
	+		xxx	xxx
htau23	-	xxx		
	+		xxx	xxx
K3M	-			
	+		(x)	
K2	-			
	+		xxx	
K17	-	xxx		
	+			xx
K10	-			
	+		xxx	xxx
K19	-			
	+			
htau40/2235	-	(x)		
	+		xxx	xxx
htau40/2396	-	xxx		
	+		xx	xxx
htau40/2235/2396	-	(x)		
	+		xx	xxx
htau23/2204	-	xxx		
	+		xxx	xxx
htau23/2396/2204	-	xxx		
	+			xxx
K2	-	xxx		
	+			xx
K5	-	xxx		
	+		xx	xxx
K5	-	xxx		
	+		xx	xxx
K7	-	xxx		
	+		x	xx
K13	-	xxx		
	+			xx
K14	-	xxx		
	+			xx
K15	-	xxx		
	+			xx